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Cell Sorter Cleaning Practices and Their Impact on Instrument Sterility

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ABSTRACT

Cells isolated using electrostatic cell sorters are subsequently evaluated in a variety of in vitro and in vivo applications. Thus, manipulations to the cells during the pre- and post-sort processing as well as when the cells are being analyzed by and passing through the sorter fluidics has the potential to affect the experimental results. There are many variables to consider when seeking to preserve cellular integrity and function during the cell-sorting process. A previous study by the Association of Biomolecular Resource Facilities Flow Cytometry Research Group (FCRG) investigated downstream effects on different cell types as a function of sorting variables such as pressure, nozzle size, and temperature. This multisite study revealed site-to-site variability based on differential gene expression when the same cell type and sort conditions were used. These results indicated the possibility that environmental factors such as the presence of contaminants in the sorter fluidics could exhibit effects on downstream molecular assays (ie, endotoxins or RNases). In the study described here, the FCRG sought to better understand how sorters are maintained and evaluated for contaminants such as bacteria, endotoxin, and RNases. In addition, the efficacy of an endotoxin decontamination method was evaluated. The results demonstrated that the majority of sorters in shared resource laboratories are free of RNase activity and bacteria; however, many are contaminated with endotoxin. The efficacy of a hydrogen peroxide cleaning procedure was tested and found to exhibit only a short-term effectiveness in eliminating endotoxin contamination.

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INTRODUCTION

Cell sorting has become a vital part of the workflow for many in vitro and in vivo assays, including coculture, adoptive transfer experiments, and the genomic or

proteomic characterization of a single cell (eg, to assess heterogeneity in a tumor). For these downstream applications, it is highly desirable for the sorted cells to exhibit minimal perturbations to their native functional state. Thus, the exposure of cells within the sorter to factors that may alter their native state has the potential to affect the function of the sorted cell population(s) (ie, cytokine production, proliferation) and ultimately affect experimental results, especially when sorting to single cells.[1],[2],[3],[4],[5],[6],[7],[8]

In any laboratory, experiments must be performed in controlled, clean environments to minimize the alteration of results because of external factors. Common laboratory biological contaminants include bacteria, molds, yeasts, and mycoplasma. Endotoxin (lipopolysaccharide; LPS), a derivative of the outer membrane of Gram-negative bacteria, is a large molecule consisting of a lipid and O-linked polysaccharide and is typically released upon the death of the bacteria. LPS is known to elicit an immune response even at very low levels (0.02 ng/mL).[9] Macrophages, dendritic cells, and B cells are particularly sensitive to endotoxin, and therefore, the presence of endotoxin in immunological studies is of particular concern. LPS activates these cells by binding to a receptor complex made up of TLR4, CD14, and MD-2. If these cell types are passed through a sorter that is contaminated with endotoxin, they could become inadvertently activated, thus affecting the results of downstream assays.

Some cell-sorting facility managers or cytometer operators have expressed concern that RNase within the instrument's fluidics may interfere with RNA-based assays downstream from cell sorting. For example, single-cell RNA sequencing (scRNA-seq) technology is based on preserving small amounts of nucleic acid material. scRNA-seq is generally known to exhibit some level of bias and degradation of quality control metrics in the presence of significant message degradation in the sample.[10] The presence of RNase in sorters is thought likely because it is environmentally ubiquitous and is often produced by microbial contamination and biofilms. However, no clear evidence in the field exists to date demonstrating whether the presence of RNases in cell sorter fluidics occurs at measurable amounts or frequencies and whether this is truly a problem needing addressing. We sought to analyze sorter fluidics from multiple sites and instruments using a sensitive RNase activity detection assay to demonstrate the presence or absence of RNase activity.

Certain cell-sorter components such as in-line sheath and/or air filters are designed to eliminate or prevent contamination by common microbial agents. These filters are designed to filter out anything 0.2 µm or larger. In addition, cell sorters are maintained

with frequent sheath tank changes, daily fluidic system (sample path) cleaning, and shutdowns performed with bleach, ethanol, detergent, or a combination of the three. In a cell sorter, the fluid is moving through the fluidics and is only stagnant during times of non-use. Even with all these precautions, sorters still can become contaminated with bacteria and/or fungi.

Because of the importance of cell sorting in modern experimental workflows, the Association of Biomolecular Resource Facilities Flow Cytometry Resource Group (FCRG) sought to expand work on this topic with the larger goal of establishing best practices in cell sorting. The previous study by the FCRG investigated how cell-sorting parameters such as nozzle size, pressure, and ultraviolet light exposure influenced cellular function in a multisite study.[11] The results of that study indicated minimal perturbations to gene expression occur following cell sorting, and they are resolved after a period of rest in culture for both cell lines and primary cells. Surprisingly, when gene expression data were compared, the site where the cell sorting was carried out influenced sample clustering. This interesting observation led the FCRG to investigate site-specific environmental conditions that could contribute to this finding. The study described here sought to understand contaminants that are commonly found in cell sorters, differences in operational cleaning protocols in cell-sorting facilities across the United States, and the efficacy of a proposed cleaning procedure to minimize environmental contaminants.

MATERIALS AND METHODS

Survey methods

To collect data from the flow cytometry community on cleaning and contamination testing practices in their laboratories, the FCRG created a survey in surveymonkey.com. The survey was disseminated using the Purdue Cytometry List and the Google+ Cytometry community.

Study participants

In addition, as part of the survey, respondents were asked if they would like to provide sheath and stream samples from their sorters to the FCRG for RNase, endotoxin, and microbial testing. Eight FCRG members provided samples from 19 sorters (Table 1) and 3 additional sheath tanks. In addition, as part of the survey, respondents were asked if they would like to provide sheath and stream samples from their sorters to the FCRG for RNase, endotoxin, and bacteria testing. Eight survey respondents sent sheath and stream samples. Additionally, 3 instruments from a FCRG member only had

stream samples submitted in the first round, so paired tank and stream samples were submitted in the second round of testing. Another FCRG member submitted tank and stream samples from an instrument not tested in the first round for a total of 28 sorters in the second round of testing (Table 1).

Table 1

Number and instrument model assayed for the presence of RNAses, endotoxin, and sterility

Instrument	FCRG member facility (*n* = 8)	Survey respondent facility (*n* = 8)
BC Astrios	2	4
BC MoFlo	2	1
BD FACSAria	12	16
BD FACSAria Fusion	0	1
BD FACSJazz	0	1
BD Influx	1	2
BioRAD Avalon/S3	2	1
Sony SY3200	0	2

A total of 47 sorters were tested; 19 sorters in round 1 and 28 sorters in round 2.

Sample collection

All participating sorters were started up following standard protocols for the respective laboratories and instruments. A sterile pipet was used to collect 2 samples (10 mL each) from the sheath tank connected to the instrument. An additional 2 samples (10 mL each) of sheath fluid were taken from the fluid stream exiting the nozzle. For instruments that internally mixed water and concentrated sheath, 2 samples (10 mL each) were collected from each of the water and concentrated sheath tanks. One sample from each source was shipped on wet ice or with freezer packs to 2 testing laboratories, 1 for analysis of bacteria (and fungus for the FCRG samples) and 1 for endotoxin and RNase levels.

Microbial detection by flow cytometry and culture

For FCRG members' samples, the presence of Gram+/− bacteria and fungus were assessed using a Cell Culture Contamination Detection Kit (Molecular Probes, catalog number C-7028) according to the manufacturer's instructions with minor modifications. Briefly, 8.0 mL of each sample was centrifuged at 10,000 × *g* at 4°C for 10 minutes. The supernatant was discarded, leaving only 200 µL of liquid with the centrifuged sample. A total of 10 µL 1:20 dilution Calcofluor White reagent (identifies fungal cell walls) was added to 100 µL resuspended sample for the detection of fungus. The remaining 100 µL sample were incubated with 50 µL 1:20 dilution Wheat Germ Agglutinin-Texas Red (WGA-TR) reagent (lectin that binds N-acetylglucosamine and N-acetylneuraminic acid residues, identifies Gram+ bacteria) followed by incubation with 2 µL of SYTO 9 reagent (nucleic acid stain, identifies Gram+/− bacteria). Both fungus and bacteria test samples were then incubated at room temperature in the dark for 5 minutes. *Escherichia coli*, *Bacillus subtilis*, and an unidentified mold fungus from bread were used as positive controls for Gram+, Gram−, and fungus, respectively. Flow cytometry analysis was performed on a MACSQuant VYB cytometer (Miltenyi Biotech). Calcofluor White was excited by a 405-nm laser and detected using a 440/40 bandpass filter. WGA-TR was excited by a 561-nm laser and was detected with a 615/20 bandpass filter. SYTO 9 was excited by a 488-nm laser and was detected using a 530/30 bandpass filter. Data were acquired and analyzed using MACSQuantify v2.5 software. To verify that the samples showed no bacteria detectable by culture, 1 mL each sample was cultured on Lysogeny broth (LB) with no antibiotics added and incubated at 37°C for 72 hours. In addition, 1 mL from each sample was cultured in 9 mL Roswell Park Memorial Institute medium (RPMI) + 10% fetal calf serum with no antibiotics added and incubated at 37°C/5% CO_2 for 5 days.

For samples from survey respondents, bacterial contamination was detected using a method developed at the University of Rochester Medical Center Flow Cytometry Resource for prospective contamination testing of sorter sheath fluid. The method is an optimized protocol for use of the FACSMicroCount reagents (BD Biosciences/BD Diagnostics) in a conventional flow cytometer with a red laser and standard APC filters (CYTO 2012 Poster Abstract #231). The reagents include the Biomass reagent (catalog number 245173), which stains nucleic acids, and the BRAG3 reagent (catalog number 245163), which reduces background. Briefly, 10 mL each sample was centrifuged at 400 × *g* at 4°C for 10 minutes. The supernatant was discarded, leaving 1 mL liquid with each centrifuged sample. The remaining sample was vigorously mixed prior to staining with 100 µL of Biomass reagent. The stained sample was gently mixed and

incubated at room temperature for 3 minutes before 100 µL of BRAG3 was added. The sample was again mixed gently and incubated at room temperature for at least 6 minutes. After incubation, the sample was analyzed using a 4-laser (407, 488, 532, and 633 nm), 18 fluorescent parameter LSRII (BD Biosciences) using FACS DIVA version 6.1.3. The Biomass reagent was excited by a 640-nm laser and detected using a 670/14 bandpass filter. Each sample was run for approximately 2 minutes, which equates to approximately 50 mL.

Endotoxin detection

For all samples, endotoxin levels were measured using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher, catalog number 88282) following the manufacturer's instructions. The kit has a detection range of 0.01 to 0.1 ng/mL LPS. All standards and samples were run in duplicate. Briefly, 50 µL standard (0, 0.1, 0.25, 0.5, and 1.0 EU [endotoxin unit of activity]/mL) or sample were added to a 37°C preequilibrated microplate and incubated for 5 minutes at 37°C followed by the addition of 50 µL of the proenzyme limulus amebocyte lysate (LAL). After incubation with LAL for 10 minutes at 37°C, 100 µL chromogenic substrate was added, and the plate was incubated for 5 minutes at 37°C. Finally, 50 µL of 25% acetic acid was added to stop the reaction. Absorbance was measured on a Biotek Synergy Microplate Reader at 405 to 410 nm. The average absorbance of the blank replicates was subtracted from the average absorbance of each standard and sample. A standard curve was calculated to determine the endotoxin concentration of each of the fluidic samples.

RNase levels

The RNase activity was determined using the RNaseAlert Lab Test Kit (Thermo Fisher Scientific, catalog number AM1964) following the manufacturer's instructions. In brief, 45 µL of each sample was pipetted into a 96-well plate. To each well, 5 mL of 10× RNase A laboratory test buffer and 5 µL of fluorescent substrate were added. A Biotek Synergy Microplate Reader (490 nm excitation and 520 nm emission) was used to measure real-time fluorescence every 5 minutes for 1 hour. RNase-free water was used as a negative control, and diluted RNase A was the positive control. All samples reached a plateau in fluorescent signal at 20 minutes incubation. According to the manufacturer, a positive sample should have 2- to 3-fold more fluorescence than the negative control, although usually a contaminated solution will be 20- to 100-fold greater than the negative control.

Data access

Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1652.

H_2O_2 endotoxin cleaning

To clean endotoxin from 4 positive cytometers (3 Arias and 1 MoFlo at 2 sites), 1% H_2O_2 was loaded into the sheath tank, and a fluidics system startup was performed. A sample tube containing 1% H_2O_2 was loaded onto the sample holder, and the stream was turned on. For FACSAria instruments, the clean flow cell procedure was performed 3 times; for MoFlo instruments, the debubble procedure was performed for 30 seconds. Then, 1% H_2O_2 was run at a flow rate of 11.0 (FACSAria) or a pressure differential of 5.0 (MoFlo) for 2 hours. The sheath tank was then thoroughly rinsed and filled with sterile H_2O or sheath fluid. Fluidics startup was performed again (2 to 3 times for the FACSAria), and a sample with sterile H_2O was loaded. The clean flow cell procedure was performed 4 to 5 times (FACSAria), or the system was debubbled for 30 seconds (MoFlo). Sterile H_2O was run through the sample line as before for 2 hours before instrument shutdown was performed.

RESULTS

To understand common contaminants of cell sorters and how they are maintained, a survey was designed and distributed to the flow cytometry community. Respondents were asked about instrument age, operation requirements, service methodology, testing procedures, and sterilization procedures (see Supplemental Fig. S1 for the full survey). Some respondents opted to send samples for bacterial contamination testing, RNase activity testing, and endotoxin testing of the fluid stream and sheath tank from their instrument(s).

Survey participants' metrics

The survey was disseminated as described in Materials and Methods. The respondents came from 61 institutions and described 106 sorters. Of those, 58 respondents operated instruments in Shared Resources Laboratories (SRLs) and 3 in single-investigator laboratories. About half of the sorters (51%) were housed in biosafety cabinets or biobubbles, and 63% were operated solely by SRL staff, whereas 29% were operated by both staff and users with a total of 37% of the sorters allowing user operation. The average usage of all sorters was relatively high, with 42% of the instruments being utilized 76% to 100% (based on a 9 am to 5 pm day), 3% at over

100%, and 27% between 51% to 75%. The age of the sorters ranged from less than 1 year to 18 years with 37% being less than 5 years of age and 43% between 5 and 10 with a total average of 6 years (Figure 1A). A majority (74%) of the instruments were covered by manufacturer service contracts. Commercial sources of sheath were used in 86% of the sorters, whereas 14% of respondents made their own sheath fluid.

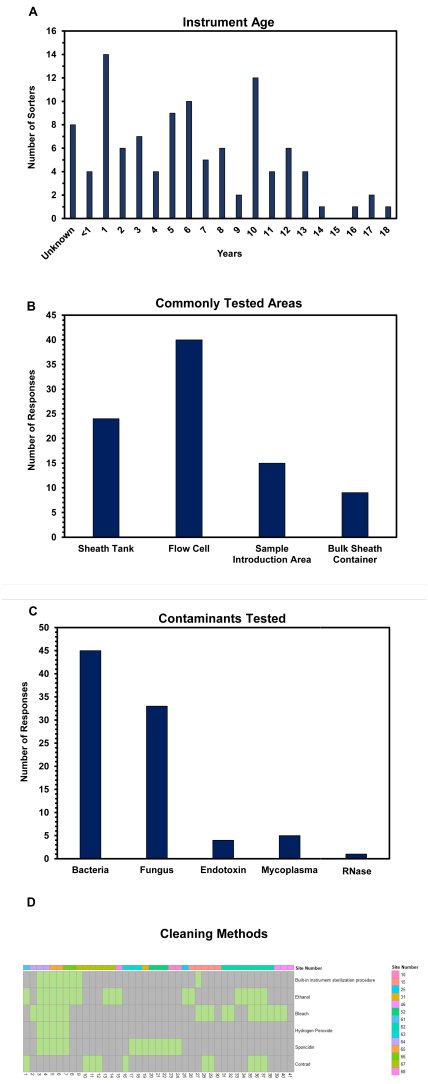

Figure 1

Responses to the survey. (A) Bars represent the number of sorters of a given age in the labs of the survey respondents. The respondents' answers were rounded up to the nearest whole number except for those whose age was less than 1 year. The unknown category was used for those sorters whose age was not given or the respondent indicated they did not know. (B) Areas that are commonly tested for contamination. Bars represent the number of positive responses for each area. The total number of responses was 88, as multiple responses from each survey participant were allowed. (C) Possible contaminants that are included in testing sorters for contamination. Bars represent the number of positive responses for each type of contaminant. Total number of responses was 87. (D) Sorter cleaning methods used in SRLs. Instrument numbers are listed across the bottom of the map. Green boxes indicate cleaning methods used by each site.

Testing for contaminants varies greatly

Because bacterial and RNase contamination of a sorted sample can have a detrimental effect on downstream assays, knowing how "clean" the sorter prior to use is very important. When survey respondents were asked a series of questions related to testing and cleaning of the sorters in their facility, 62% reported that they test only when contamination is reported by users or do not test at all (Table 2). Most respondents tested the flow cell in their sorters and the attached sheath tank, but very few tested the bulk sheath supply (Figure 1B). All 45 laboratories that reported contamination testing of some kind indicated they included testing for bacterial or fungal contamination. Only 4 laboratories indicated testing for endotoxin. Only 1 respondent indicated they tested for RNases (Figure 1C).

Table 2

Self-reported testing practices

Frequency	Responses (*n* = 53)	Percentage
Daily	2	4%
Weekly	11	21%
Monthly	7	13%
Never	8	15%
Only upon possible contamination	25	47%

A total of 47 individuals responded to the question about how many incidents of contamination they had in the past 12 months, including 2 individuals who never test their sorters. Whereas 21 (45%) of the respondents had no contamination, 17 (36%) had only 1 or 2 instances of contamination. Surprisingly, 3 of the respondents reported 5 or more instances of contamination, 1 of whom reported more than 10 instances. Most reported contamination was bacterial. The most common way respondents cleaned their sorters was with bleach, ethanol, and/or built-in instrument sterilization processes (Figure 1D).

Assessing the presence of bacteria and fungus in the fluidics of cell sorters

To test for common laboratory contaminants, 8 FCRG members submitted a total of 38 samples collected from the sort stream and sheath tank of their cell sorter(s). All samples were sent to a single laboratory and stained using the Cell Culture Contamination Detection Kit. The positive controls show positive bacteria and fungus populations distinct from the negative population (Figure 2A). Representative microscopic images of positive controls versus samples are shown in Figure 2B. All the samples tested were at or below background except for 2 (circled in red; samples 15 and 23; Figure 2C). To confirm the results, aliquots from each sample were cultured in LB broth or RPMI for 3 or 5 days, respectively (data not shown). Microscopic evaluation of sample 15 found no evidence of microbial contamination. Culturing aliquots of sample 15 for 2 additional days in RPMI and 3 additional days in LB broth showed no growth and were negative upon restaining and reanalysis (data not shown). Microscopic evaluation of sample 23 found a precipitation of crystals (data not shown), likely because of the salt content in the sheath.

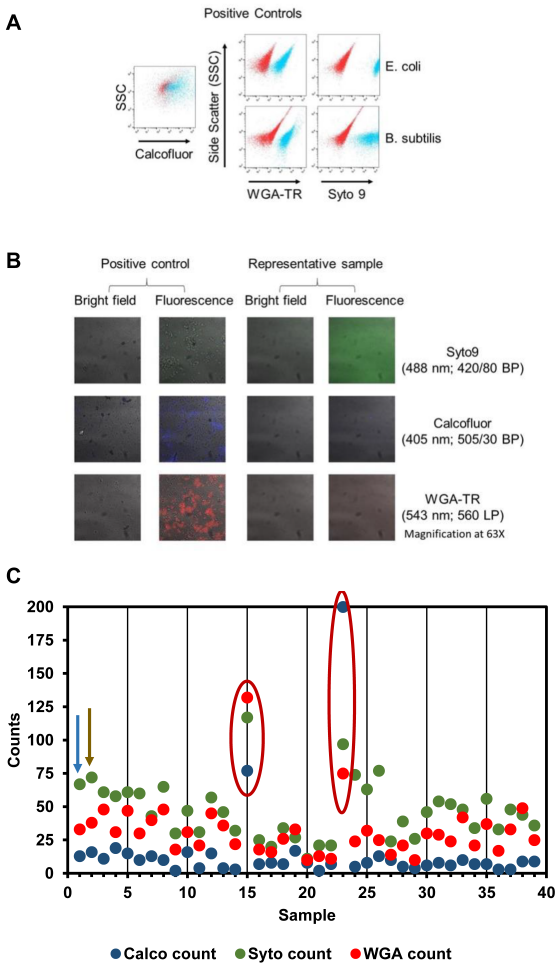

Figure 2

FCRG members' sorters and sheath tanks were free from microbial contamination. The Thermo Fisher Molecular Probes Cell Culture Contamination Detection kit was used to assess contamination. (A) Dot plots showing the staining of the positive control bacteria (E. coli and B. subtilis) with calcofluor (stains fungi), WGA-TR (stains Gram(+) bacteria) and Syto9 (stains Gram(+) and Gram(-) bacteria). (B) Images taken on a fluorescent microscope showing bright field and fluorescence staining of the positive control bacteria and a representative test sample. (C) Graph showing the number of counts for each sample tested. Blue arrow is the water negative/background control sample. Brown arrow is the PBS negative/background control sample. For each sample, blue dots indicate calcofluor(+) results, red dots indicate WGA-TR(+) results, and green dots indicate Syto9(+) results. Two samples with positive results indicated by red ovals. Note: The sample numbers do not correspond to the instrument numbers in other figures.

Survey respondents from 10 laboratories sent samples from both sort streams and sheath tanks to an FCRG member laboratory for microbial contamination testing,

which assessed the presence of both bacterial and fungal components. It should be noted that some of the survey respondents that sent samples for RNase activity and endotoxin testing did not include samples for microbial testing. Figure 3A shows dot plots of representative samples demonstrating negative, low-frequency positive (“+”), mid-frequency positive (“++”), and high-frequency positive (“+++”) microbial contamination. Of the 17 sorters tested, 18% of the sort streams were highly contaminated (+++), whereas 71% were negative for microbial contamination (Figure 3B). Interestingly, whereas only 30% of the streams had any microbial contamination, 88% of the sheath tanks tested positive (Figure 3B). The lower frequency of contamination in the stream demonstrated the efficacy of the standard 0.2-µm in-line sheath filter, which removes most microbial agents from the instrument fluidics path.

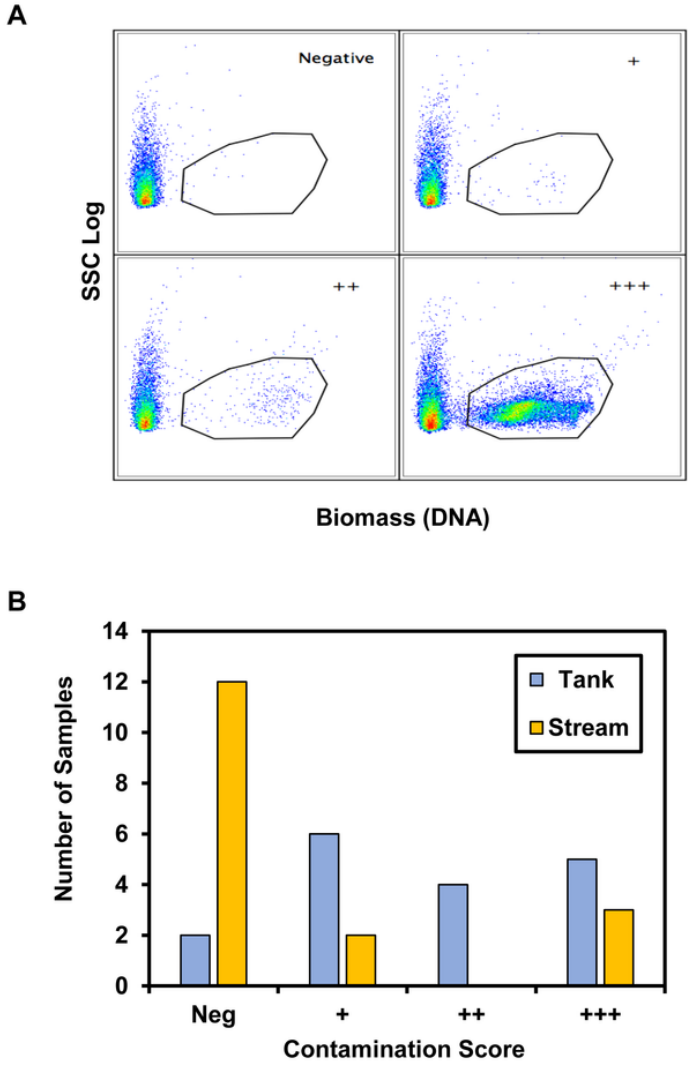

Figure 3

Screening survey participants' sorters and sheath tanks for bacterial contamination. The BD Biosciences FACS MicroCount™ kit was used to detect bacterial contamination in the samples. (A) Dot plots show example negative, +, ++ and +++ contamination scores. The gate in each graph identifies the bacteria. (B) Cumulative results of the testing of 17 sheath tank and 17 sorter stream samples. Blue bars indicate the results from the sheath tank. Gold bars indicates results from the sorter stream.

Majority of sorters are not contaminated with active RNases

RNA is often isolated from sorted cells for analysis in applications like scRNA-seq. Thus, the presence of RNases could impact the quality of results for these types of assays that rely on the presence of intact nucleic acids. Although testing sorters for RNases is not routinely done, even in SRLs, the FCRG decided to test the sorters and sheath tanks in their facilities for RNase activity (Figure 4A). In addition, several survey respondents sent samples for RNase activity testing (Figure 4B). Only 3 out of the 96 samples tested (3.1%) had a fluorescence level greater than 2 times the negative control. Of the 3 positive results, only 1 was slightly higher than 3 times the negative control. As RNase-contaminated solutions are typically 20- to 100-fold greater than the negative control, it would be prudent to retest the instruments using fresh samples to confirm the results. An analysis of cleaning methods and RNase results showed no correlation (Supplemental Fig. S2). This is not surprising considering most of the RNase data were essentially 0 and not above the assay's limit of detection. Overall, these data indicate little to no active RNases in sorters and sheath tanks, and if degraded/poor-quality RNA is isolated from sorted cells, it is likely because of another source.

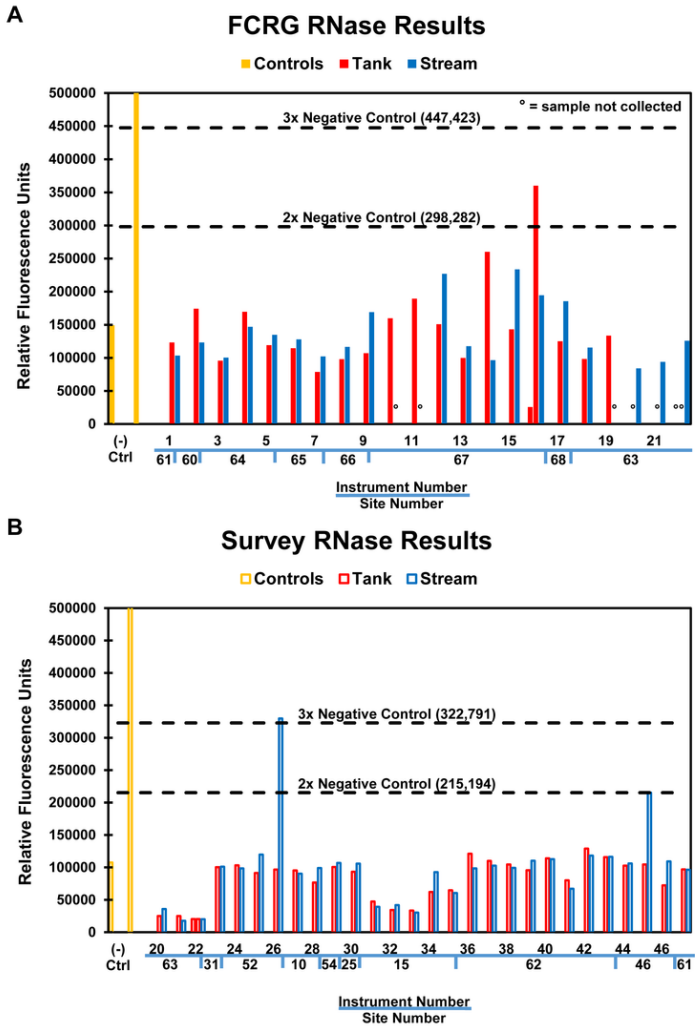

Figure 4

Measurement of RNase activity in the sorters' streams and sheath tanks. RNase activity was measured using Ambion RNaseAlert® Lab Test kit. For both (A) and (B), yellow bars are the controls, red bars represent the results from sheath tanks, and blue bars represent the results from sorter streams. The negative control was RNase-free water and the positive control was RNase-free water spiked with RNase A. Dotted lines are the 2x (lower) and 3x (upper) the negative control values. Instruments 16 and 22 contained internal sheath mixing systems; the first columns are water tanks, the second columns are concentrated sheath tanks, and the third columns are streams. (A) Graph of the measured RNase activity in FCRG members' sorters and sheath tanks. The positive control value is 8,471,805, which is off scale on the graph. (B) Graph of the measured RNase activity in survey respondents' sorters and sheath tanks. The positive control value is 5,423,458, which is off scale on the graph.

Many sorters are contaminated with endotoxin

Unlike the bacterial contamination and RNase activity assays, most sorters tested were positive for endotoxin (Figure 5). This was true for the FCRG members (Figure 5A), in which 63% of the tested sorter streams were positive, as well as for the survey respondents (Figure 5B), whose sorter stream samples were 79% positive. The results indicate a significant level of potential endotoxin contamination is endemic in cell sorters in the cytometry community.

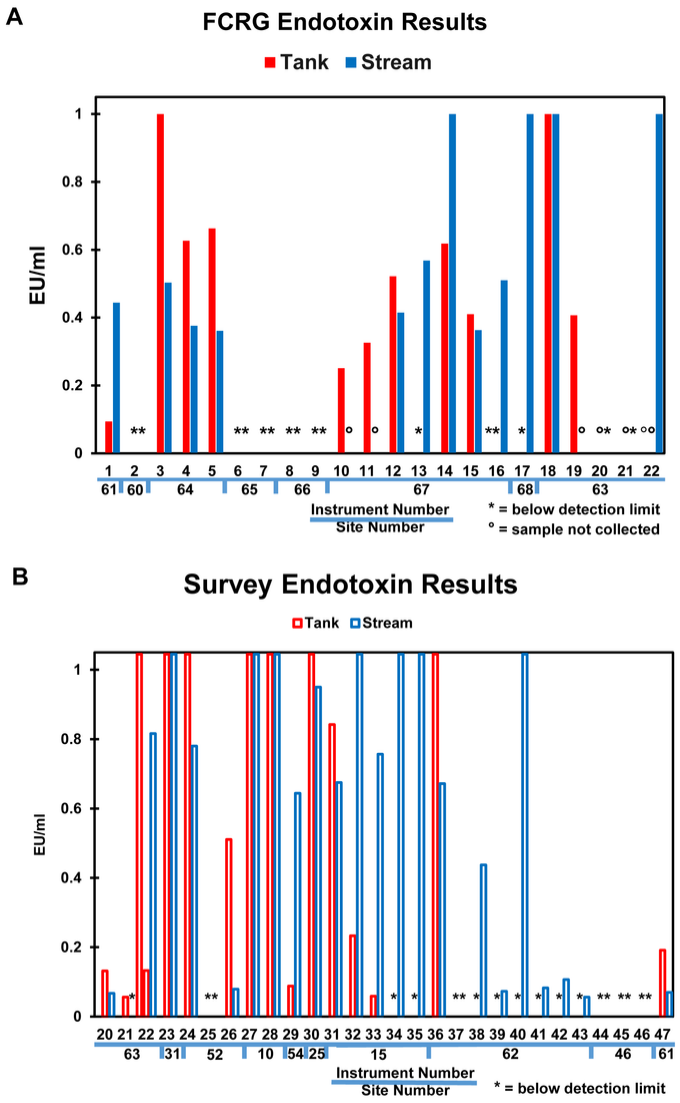

Figure 5

Endotoxin levels in sorters' streams and sheath tanks. The Pierce LAL Chromogenic Endotoxin Quantitation kit was used to measure endotoxin levels. Red bars represent the results from sheath tanks. Blue bars represent the results from sorter streams. Instruments 16 and 22 contained internal sheath mixing systems; the first columns are water tanks, the second columns are concentrated sheath tanks, and the third columns are streams. (A) Graph of the measured endotoxin level in FCRG members' instruments; the minimum detection level was 0 EU/ml and the maximum detection level was 1 EU/ml. (B) Graph of the measured endotoxin level in the survey respondents' instruments; the minimum detection level was 0.055 EU/ml and the maximum detection level was 1.045 EU/ml.

Instrument age and the presence of endotoxin contamination were not correlated. Other variables culled from the survey results that were compared with endotoxin

contamination included the following: the source and type of sheath fluid used, known recent contamination, the date of the last preventative maintenance check, cleaning methods, modifications to the instrument's fluidics system, and whether bacteria or yeast is run through the instrument. None showed significant correlation (data not shown). Further statistical analysis indicated that there were factors influencing endotoxin values that were not captured in the survey (Supplemental Fig. S2). In one laboratory, it was later discovered that a subset of sheath carboys, which are autoclaved before each use, were heavily contaminated with endotoxin at high levels (private communication).

It is accepted by the research community that 1 EU is equivalent to approximately 0.1 to 0.2 ng/mL (www.sigmaaldrich.com/technical-documents/articles/biology/what-is-endotoxin.html). In the literature, it has been demonstrated that as little as 0.002 to 2 ng/mL endotoxin can affect human immune cells.[9] Thus, the level of endotoxin in most of the positive sorter streams and sheath tanks has the potential to activate cells during the sorting process and represents a topic that the community and vendors may wish to address systematically.

H_2O_2 demonstrates effective but short-term clearance of endotoxins

Because endotoxin has the potential to activate cells, 4 sorters (3 BD FACSArias and 1 MoFlo) located in 2 different SRLs were cleaned with a diluted (1%) hydrogen peroxide solution using a protocol based on a BD Application Note by McIntyre and Reinin (https://www.bdbiosciences.com/documents/FacsariaII_Endotoxin.pdf) and articles by Sandle[12] and Lin et al.[13] Although all instruments were endotoxin free based on the testing of samples collected the day after cleaning, within 3 to 10 days, all subsequent samples tested positive for endotoxin (data not shown). This data demonstrates both the pervasiveness and persistence of endotoxins as a common laboratory contaminant.

DISCUSSION

A common concern in cell sorting is contamination of the fluidics system with agents such as bacteria, fungus, endotoxins, and/or RNase. Sheath fluid and samples introduced to the instrument are the predominant sources of contamination. The most frequently used sheath fluids are saline solution (SS) and phosphate buffered saline (PBS); however, the source of sheath fluids is quite diverse among SRLs and includes ready-to-use 1× SS or PBS, diluted-in-house 10× SS or PBS, and made-in-house 1× SS or PBS. Some SRLs minimize contamination risk by filling the sheath tank in a sterile

hood, but not all are able to do so. Survey results showed that most SRLs choose to trust their sheath fluid and standard fluidics cleaning procedures until a user returns with a complaint about contamination. Because not all types of contamination are obvious in downstream results, it is important for the flow cytometry community to be cognizant of the possible effects and proactive in the prevention of contamination.

Often, sorted cells are utilized for both in vitro and in vivo assays. In such experiments, bacterial or fungal contamination from the sorter could conceivably introduce confounding variables or prevent proper growth or function of cells. Although results from FCRG members' instruments indicated bacterial and fungal contamination below detectable levels, the broader community's samples revealed that a large percentage of common-use sorters do have detectable bacterial levels. As such, the sterilization of sheath fluid and cleaning or even replacement of instrument tubing and filters before sorting cells for culture, adoptive transfer, or similar experiments are recommended.

An important application for cell sorting is the examination of gene expression profiles of purified cells via scRNA-seq and other methodologies. In such experiments, the presence of RNase contamination in a cell-sorting instrument could have significant detrimental effects on the downstream results. Testing for RNase activity found that less than 4% of tested samples had RNase levels 2 to 3× above the negative control, suggesting that even the few sorters that do have measurable contamination only have low levels.

Endotoxin testing found detectable levels in more than 60% of the sorter streams and sheath tanks tested. The level of endotoxin found in most instruments was sufficient to cause activation of immune cells.[9] To determine whether endotoxins correlated with any instrument variables, we compared endotoxin levels to a variety of parameters collected in our survey, including age, sheath source, sample types, cleaning methods, and instrument maintenance. None of the variables predicted the presence or extent of endotoxin contamination.

A standard endotoxin cleaning protocol was tested on 4 contaminated sorters (3 located at one FCRG member's facility and 1 located at another FCRG member's facility) and the sheath tanks connected to the instruments. The protocol involved using H_2O_2 to flush the sorter's fluidics lines for 2 hours followed by rinsing the lines with water for approximately 3 hours until the H_2O_2 was cleared out. H_2O_2 was used because it oxidizes the endotoxin, thereby inactivating it.[13] Although endotoxins were below the detection limit for most tested instruments immediately after cleaning,

they returned to precleaning levels within 3 to 10 days, suggesting bacteria and/or endotoxins are reintroduced through outside sources. Because the H_2O_2 cleaning protocol takes over half a day to complete, a requirement that can be quite difficult to fit on a busy sorter's (and staff member's) weekly schedule, we recommend that cell sorters be cleaned immediately prior to those specific experiments that are particularly susceptible to endotoxin contamination. Without a clear source or cause, it is particularly difficult to implement measures to prevent contamination.

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Supplemental Material

Sterility Paper Supplemental Figures 111021.docx 125 KB

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